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(57) Abstract

The invention relates to a biodegradable construct comprising chondrocytes and a chitosan–based composite material which is complexed with a glycosaminoglycan molecule. Also, a method of transplanting chondrocytes to a patient can be accomplished by placing chondrocytes in the construct which comprises a chitosan material which is complexed with a glycosaminoglycan molecule and placing said construct containing said chondrocytes in said patient. Additionally, damaged articular cartilage can be repaired by administering, to the site of said damage, a therapeutically effective amount of chondrocytes in a construct comprising the chitosan–based composite material which is complexed with a glycosaminoglycan molecule.

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CHITOSAN-BASED COMPOSITE MATERIALS CONTAINING GLYCOSAMINOGLYCAN FOR CARTILAGE REPAIR

BACKGROUND OF THE INVENTION

The present invention relates generally to methods of healing or repairing cartilage by transplantation of chondrocytes, particularly articular cartilage. Specifically, the invention relates to tissue engineering which involves the guided growth of autologous cells to replace damaged or attenuated organs and structures. The present inventors have devised chitosan-based composite materials which contain glycosaminoglycan in the form of a construct, that closely mimics the biochemical environment of cartilage, for transplantation of chondrocytes.

Cartilage is the specialized, fibrous connective tissue, forming most of the temporary skeleton of the embryo, providing a model in which most of the bones develop, and constituting an important part of the growth mechanism of an organism. Articular cartilage (AC) is a thin layer of cartilage, usually hyaline, on the articular surface of bones in synovial joints. Once damaged, in general, cartilage has very little capacity for spontaneous healing. The inability of cartilage to heal itself and the clinical need for long-lasting repair of cartilage lesions has been recognized for centuries. With cartilage injury, an affected joint is predisposed to undergo osteoarthritic degeneration. Osteoarthritis affects 2% of the total U.S. population of persons between the ages of 25 and 74. The knee joint is most commonly affected. Without intervention, patients with this condition often develop debilitating pain and eventually require total joint replacement. A variety of surgical techniques have been developed to repair the damaged joint. Abrasion arthroplasty, excision and drilling, cartilage debridement, and arthroscopic shaving have been used to evoke repair responses in which the damaged site is then filled with fibrocartilage. Using fibrocartilage is advantageous in some aspects but it lacks the durability and many of the properties of normal cartilage. Typically, fibrocartilage degenerates over time, resulting in clinically significant symptoms. As a result, severely symptomatic patients with cartilage defects often require further reconstructive surgery, and eventually a joint prothesis or replacement.

An alternative to typical surgical techniques involves transplantation of perichondrial grafts and periosteum. This technique has produced some encouraging results in animal models. These techniques are limited, however, by the availability of donor tissue, poor graft integration, and potential for graft ossification. Osteochondral autografts and allografts also have been used to treat osteochondral lesions successfully. But they are similarly limited by the availability of donor tissue and graft integration. Additionally, osteochondral transplant is an aggressive option for treatment of simple chondral injury.

More recently, implantation of autologous, cultured chondrocytes for the treatment of full-thickness cartilage defects has been reported to effect joint surface repair. The long term outcome of this technique, however, is unpredictable due to several factors, including the lack of internal fixation of the graft. Tissue-engineering concepts have been applied to meet the challenge of graft fixation within chondral defects. As a result, a variety of biomaterials have been developed to create chondrocyte-seeded implants. These materials include collagen gels, collagen fibers, demineralized bone matrix, polyglycolic acid, polylactic acid, and fibrin. For example, a type I collagen-based gel is biocompatible and has been suggested as a three-dimensional scaffold for damaged cartilage. However, a chemical compositions of type I collagen-based gel does not approximate the *in situ* environment of articular cartilage very well because the in situ environment of articular cartilage is type II collagen. Thus, type I collagen gels fail to produce reliable results in most *in vivo* animal models. Foreign protein-based substances, such as collagen gel, lack immuno-compatibility and possibly cause an immune response *in vivo*. Fibrous polyglycolic acid (PGA) scaffolds are biocompatible and biodegradable, but they produce an acidic residue during the degradation process, which can exhibit toxic effects on seeded cells. Mechanically strong synthetic implants like those composed of carbon fiber mesh coated with hyaluronic acid also have been suggested. However, it is known that carbon fibers evoke a chronic inflammatory reaction *in vivo*, which leads to joint synovitis. Both alginate and agarose polysaccharide gels have demonstrated excellent chondrogenetic characteristics for chondrocyte cultures *in vitro*. However, they are not well suited for clinical implantation into human cartilage, because they are not biodegradable. Some of these biomaterials exhibit a short term formation of repair tissues resembling hyaline cartilage, the long term results suggest inherent limitations of these carrier substances in the clinical applications for repair of damaged articular cartilage.

The present invention develops a method of using chitosan as a chondrogenic carrier vehicle of chondrocytes for the purpose of chondrocyte transplantation in surgical repairs of damaged cartilage. Chitosan is the partially de-acetylated derivative of chitin, found in the exoskeletons of most arthropods, and is second only to cellulose as the most abundant naturally-occurring polymer. Structurally, chitosan is a polycationic linear polysaccharide, a repeating monosaccharide of β-1,4 linked glucosamine monomers with randomly located N-acetyl glucosamine units, and thus shares characteristics similar to glycosaminoglycan and hyaluronan. Chitosan-based materials have previously been used to immobilize or encapsulate mammalian cells for the three-dimensional growth of a variety of tissue types including liver (Kawase *et al, Biol. Pharm. Bull.*, 26:708-710, 1997), and blood vessels (Madihally *et al*, ASME Bioengineering Conference, BED-Vol. 35:417-418, 1997). Zielinski *et al.*, *Biomaterials* 15: 1049-56 (1994), have demonstrated the use of a chitosan matrix which comprises cationic chitosan and anionic sodium alginate. In this *in vitro* study, the biological behaviors of three different types of cells (PC12, R208F, R208N.8) were examined in a precipitated chitosan matrix. The study showed that a cationic hydrogel, precipitated chitosan, provided cell viability and supported attachment and spreading of these cells.

Due to its polycationicity, chitosan can be electrostatically crosslinked with anionic glycosaminoglycan molecules to form a hydrogel. Use of the GAG-augmented chitosan substance to develop a cell-based repair material for articular cartilage has many advantages including biocompatability, biodegradability, low immunogenicity, and low cost. This substance can be readily modified to a variety of forms including a membrane, a bead, or a 3-dimensional porous scaffold, which may be directly implanted to deliver laboratory-cultured chondrocytes into cartilage lesions in joints. It is expected that the intrinsic chondrogenic characteristics of the GAG-augmented chitosan hydrogel promote the metabolic activities of transplanted chondrocytes over the course of repair process of damaged cartilage. Even though, the GAG-augmented chitosan hydrogel exhibits some structural stiffness it is preferable to supplement the GAG-augmented chitosan to increase structural stiffness to to able to withstand a significant amount of mechanical stress which may occur during articulation of the joint post-operatively. Therefore, an improvement in the mechanical properties of the chitosan backbone has been needed.

To improve the structural stiffness of the scaffold, the present inventors form a composite structure of chitosan and a polyglycolic acid/poly-l-lactic acid (hereafter

"PGA/PLLA"), which combines the diverse functionality of the chitosan molecule with the structural stability of the bioresorbable PGA/PLLA thereby providing additional structural stiffness. PGA/PLLA has been approved by the Food and Drug Administration (FDA) for implantation in humans, and has been utilized in various applications such as biodegradable suture material, biodegradable fracture fixation devices, and biodegradable surgical screws. The mechanical properties and physiological degradation characteristics of PGA/PLLA lend it to be added to chitosan to improve the mechanical strength of the chitosan scaffold. When used as a temporary scaffold in tissue engineering to regenerate articular cartilage, the PGA/PLLA component provides a large portion of mechanical strength of the tissue engineering matrix until chondrocyte ingrowth and extracellular matrix production could supplant the mechanical stability of the gradually eroding PGA/PLLA-chitosan matrix.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a material that mimics the *in vivo* environment of cartilage, that is biodegradable and can support the culturing of chondral cells, and therefore that can act as a vehicle for chondrocytes, and that possesses a stiffness sufficient to accommodate the mechanical-strength needs of a wide range of implant applications.

It is another object of the present invention to provide an means for transplanting chrondrocytes, in order to effect cartilage repair.

It is yet another object of the present invention to provide an approach to repairing a cartilage defect, via open arthrotomy, into the defect site, of a material that supports chondrocyte proliferation and maturation.

In accomplishing these and other objects, there has been provided, in accordance with one aspect of the present invention, a construct that comprises chondrocytes and a chitosan-based composite material which is complexed with a glycosaminoglycan molecule, such as heparan sulfate, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, dermatan sulfate, keratin sulfate, hyaluronic acid, or heparin. The construct can take the form of a hydrogel, a film, a three-dimensional scaffold or a microcapsule, *inter alia*. In one preferred embodiment, the construct takes the shape of a chondral defect site in a patient. In another preferred embodiment, the chitosan- material comprises a composite of chitosan and polyglycolic acid/poly-l-lactic acid.

There also has been provided, in accordance with another aspect of the present invention, a method of transplanting chondrocytes to a patient, comprising placing chondrocytes in a chitosan-based composite material complexed with a glycosaminoglycan molecule and placing the construct containing the chondrocytes into a patient in need of such transplanting and a method of repairing damaged cartilage, comprising administering to the site of damage, a therapeutically effective amount of chondrocytes in the inventive construct.

These objects and others apparent to those skilled in the art have been achieved by the invention described below in the detailed description and appended claims.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present inventors have developed a construct of a chitosan-based composite material which is complexed with a glycosaminoglycan (GAG) molecule and which possesses the properties necessary for *in vitro* chondrocyte culturing. Thus, a chitosan-based composite material is one that comprises natural chitosan or a composite of chitosan and polyglycolic acid/poly-l-lactic acid. The construct acts as a culturing substance for chondrocytes. The construct can be in the form of a film, microcapsule or a three-dimensional scaffold. The chitosan-GAG complex material acts as a carrier material for chondrocytes, and provides a construct for *in vivo* cartilage repair. The interaction between the cationic chitosan material and anionic GAG can produce a stable 3-dimensional complex matrix, a thin membrane film suitable for chondrocyte culture, or a microcapsule for encapsulation of cultured chondrocyte cells. Chondrocytes embedded in the inventive chitosan-GAG complex material maintain viability and phenotypic characteristics of mature, differentiated chondrocytes. Chondrocytes cultured on the inventive chitosan-GAG complex material also maintain their phenotypically round shape even after attachment to the membrane for two weeks. The culture also demonstrates superior metabolic activities, producing more proteoglycan molecules as compared to standard cell culture conditions.

Chitosan, a deacetylated derivative of chitin, is a cationic hydrogel-forming polysaccharide substrate. Due to its cationicity, chitosan can be complexed with anionic GAG molecules to provide a three-dimensional matrix environment for chondrocyte culture, which can mimic the *in situ* GAG rich environment of articular cartilage.

Glycosaminoglycan (GAG) denotes any of several high molecular weight linear heteropolysaccharides which have repeating disaccharide units which contain an N-acetyl-

hexosamine residue and a hexose or hexuronic acid residue; either or both of these residues may be sulfated. The exemplary class of glycosaminoglycans are the chondroitin sulfates (A, B, and C), dermatan sulfates, heparan sulfate and heparin, keratan sulfates and hyaluronic acid. Chondroitin sulfate A is one of the major glycosaminoglycan molecules found in articular cartilage.

Polyglycolic acid (PGA) and poly-l-lactic acid (PLLA) are two synthetic polymer substances, which have been found to be biocompatible and biodegradable, and approved by the Food and Drug Administration for clinical applications. They can be used as separate substances of each or as a combined coplymer substance of (PGA/PLLA). Currently, PGA/PLLA is used in biodegradable suture materials and biodegradable fracture fixation devices. The mechanical properties and physiological degradation behavior of PGA/PLLA add improved mechanical properties to the chitosan material. When used in articular cartilage tissue engineering, the PGA/PLLA component of the chitosan-based composite material provides mechanical integrity to the tissue engineering material until chondrocyte growth and extracellular matrix production could supplant the mechanical stability of the gradually eroding PGA/PLLA-chitosan composite.

According to one embodiment of the present invention, a chitosan-GAG complex material that closely mimics the natural GAG-rich environment of cartilage, particularly articular cartilage, provides a biological vehicle for transplanted chondrocytes. Since most of the natural GAG molecules are negatively charged, GAG molecules can be complexed with a cationic chitosan material which provides a synergistic effect of various GAG molecules on chondrocyte metabolism, i.e., chondrogenesis is enhanced as a result of a GAG-rich environment which mimics the articular cartilage site.

In a preferred embodiment, the chitosan material comprises an emulsified composite of chitosan and PGA/PLLA and at least one GAG-molecule. Because it is known that PGA/PLLA materials biodegrade in vivo, it can lend these properties to the chitosan material for culturing chondral cells.

In another embodiment, the chitosan material can be freeze-dried and complexed with anionic GAG molecules to form a biodegradable, 3-dimensional scaffold for chondrocyte transplantation for cartilage repair. Furthermore, a 3-dimensional scaffold made of chitosan can be molded, virtually into any shape and used, during an early stage of repair process, as a temporary biodegradable construct in place of the chondral defect site. A 1% (weight/volume

ratio) chitosan solution dissolved in 0.2M acetic acid can be placed in a glass container, the shape of which was pre-molded to match a required geometry of the chondral defect site, rapidly frozen either in a dry-ice environment or in a liquid nitrogen environment, and then lyophilized for about 24-48 hours. The geometric shape of the chitosan material will be maintained during the lyophilization process, and the outcome of this process will be a porous chitosan scaffold with the pre-determined geometry. Once it is completely freeze-dried, the porous chitosan scaffold can then be complexed with anionic GAG molecules to produce the final product of chitosan-GAG scaffold.

In another embodiment of the invention, the composite of chitosan and polyglycolic acid/poly-l-lactic acid is freeze-dried and complexed with anionic GAG molecules to form a biodegradable, 3-dimensional scaffold possessing an average pore size of about 40 to 200 μm, for chondrocyte transplantation for cartilage repair. Furthermore, a 3-dimensional scaffold made of the inventive biomaterial can be molded, virtually into any shape, and used during an early stage of repair process, as a temporary biodegradable material in place of the chondral defect site. For example, a 2% (weight/volume ratio) PGA/PLLA-chitosan solution can be placed in a glass container, the shape of which was pre-molded to match a required geometry of the chondral defect site, rapidly frozen either in a dry-ice environment or in a liquid nitrogen environment, and then lyophilized for about 24-48 hours.

Via the above-described technique, structures of variable shapes and sizes can be prepared by freezing and lyophilizing the chitosan materials. The resultant chitosan based scaffolds would possess an average pore size ranged from about 50 to about 250 μm, and therefore, may serve as a template for chondrocyte ingrowth *in vitro* and cartilage assembly *in vivo*.

In one embodiment of the invention, the interaction between a cationic chitosan material and anionic GAG may be used to produce a stable thin membrane film for chondrocyte culture. The production of a thin film can be used to culture chondrocytes.

A further embodiment of the invention encompasses chemically modifying the terminal amino group with peptide hormones to promote chondrogenesis. For example, incorporating TGF-β1 or IGF-1 into the composite material. TGF-β1 has been reported both to stimulate proteoglycan synthesis by articular chondrocytes (Morales and Roberts, J Biol. Chem. 263:12328-12831, 1988). IGF-1 has been shown to increase matrix synthesis and

stimulate mitotic activity of chondrocytes in culture (McQuillan et al, Biochem. J. 240:423-430, 1986).

The present inventors have determined that the chitosan-based scaffold acts as a construct for chondrocytes, which are used to repair damaged articular cartilage. The present invention contemplates repair of other kinds of musculoskeletal soft tissues, such as the meniscus and intervertebral discs.

In order to achieve cartilage repair, cartilage biopsy specimens are harvested from healthy shoulder and knee articular cartilage from a less weight bearing area of the joints. Chondrocytes are then harvested from the biopsy specimens using the method described by Green, *Clin. Orthop. Rel. Res.*, 123: 237-250, (1977). Harvested chondrocytes then are grown in monolayer culture on polystyrene coated culture flasks in nutrient media. When 90% confluence is achieved, chondrocytes are then subcultured. Prior to surgical transplantation of the chondrocytes, the chondrocytes are micro-encapsulated within multiple glycosaminoglycan-coated chitosan hydrogel beads, complexed within a chitosan-GAG scaffold or complexed with a chitosan-GAG film. The microencapsulation procedure involves: dissolving chitosan salt in an acetic acid solution, adjusting the pH of the solution with an aqueous solution of NaOH to approximately pH 6.3, gently mixing a predetermined portion of chondrocyte cells with the chitosan solution, forming microcapsules as droplets of the chitosan-chondrocyte solution by air entrainment from a catheter, and collecting the microcapsules in a vigorously stirred glycosaminoglycan solution. The chondrocytes can also be precipitated and seeded within the chitosan-GAG scaffold or on the chitosan-GAG film.

The surgical technique generally involves an open arthrotomy, wherein the chondral defect site is exposed to an open air, or an arthroscopic surgery, wherein the chondral defect site is approached using arthroscope instruments through small portal holes. The defect site is filled with a chondrocytes-seeded scaffold or microcapsules, and is then sealed with a patch of chitosan-GAG film, which can be sutured onto the surrounding normal cartilage. When an arthroscopic surgery is conducted, chondrocytes-seeded microcapsules can be delivered to the defect site using a syringe with a large catheter (about 2 millimeter-in-diameter). Then the joint is allowed to heal wherein the construct of a chitosan material complexed with a GAG molecule biodegrades and the chondrocytes proliferate to reconstruct chondral defect site.

The chondral defect site includes sites with partial cartilage deterioration and complete cartilage deterioration. Also, it is possible to effect cartilage growth on a non-smooth surface, e.g., defect sites wherein calcification is present.

The following examples described below are simply used to illustrate the invention. Those skilled in these arts will recognize that variation of the present materials fall within the broad generic scope of the claimed invention.

EXAMPLES

Fresh articular cartilage is obtained from the metacarpophalangeal joints of young (1-2 year old) bovine specimens. Within four hours of sacrifice, chondrocytes were isolated from the tissue using the sequential enzymatic digestion technique described by Green, *supra.* Isolated chondrocytes are then re-suspended in a polysaccharide gel.

Example 1-Encapsulation of Chondrocytes in Alginate Gel: Low viscosity alginate powder is dissolved in 0.15M NaCl and clarified by centrifugation according to the method of Guo *et al.*, *Conn. Tiss. Res.* 19: 277-97 (1989), incorporated herein by reference. Isolated chondrocytes are suspended at a concentration of 1 x 10^6 cells/ml. The chondrocyte suspension is slowly extruded from a 3 ml syringe equipped with a 22-gauge needle into 40ml of gently agitated 102mM $CaCl_2$ to form cell-containing gelled beads. The $CaCl_2$ is decanted, the beads then are washed sequentially 4 times in 0.15M NaCl, and transferred to 6-well culture plates.

Example 2-Suspension of Chondrocytes in 1% Agarose Gel: Agarose powder is mixed with DMEM and dissolved into solution by heating to 80°C. Subsequently, the solution is cooled to 37°C and diluted with an equal volume of DMEM with 20% fetal bovine serum containing resuspended chondrocytes, and brought to a final concentration of 1 x 10^6 cells/ml according to the method of Benya *et al.*, *Cell* 30: 215-24 (1982), incorporated herein by reference. Agarose medium containing 1 x 10^6 cells, are transferred to 6-well culture plates and allowed to gel at room temperature.

Example 3-Encapsulation of Chondrocytes in Chitosan-Chondroitin Sulfate-A Hydrogel: Deacetylated chitosan is dissolved in an acetic acid solution (pH=6.3). Osmolarity of the

solution is adjusted to 300mOsm/l by additions of sorbitol. Pelleted chondrocytes are resuspended in chitosan to a final concentration of 1 x 10^6 cells/ml. Microcapsules are formed as droplets of the chitosan-chondrocyte solution are generated by air entrainment from the 3ml syringe equipped with a 25-gauge catheter and collected in the vigorously stirred 10% chondroitin sulfate-A solution (pH = 7.4, Osm=300mOsm/l). The chondroitin sulfate-A solution is decanted, the beads washed sequentially 4 times in PBS, and then transferred to 6-well culture plates.

The chondrocyte cultures prepared as described above are incubated under identical conditions in 2 ml of low-glucose HEPES-buffered DMEM supplemented with 10% fetal bovine serum.

Example 4-Preparation of Composite of Chitosan and PGA/PLLA: 50:50 PGA-PLLA crystals (Medisorb) of medical implant quality are first dissolved in chloroform to make a 7% solution. This solution is mixed with 2% chitosan (in acetic acid) in a volume ration of 1:4. The two phases are insoluble in each other so the mixture is vigorously shaken and then placed in an ultrasonic bath for one minute to prepare a well-distribute emulsion. This emulsion is then loaded into a glass bone-biopsy syringe and extrusion cast into a freezing mold. The mold is then placed in a -150°F freezer for one hour to quick freeze the preparation. After freezing, the mold is lyophilized for 24 hours to remove all liquid phases and lock the scaffold in its 3D shape. The result is a highly porous, well mixed chitosan-PGA/PLLa biomaterial application. The cationic chitosan-salt portion of the structure is complexed with the highly anionic chondroitin sulfate-A, washed repeatedly with phosphate-buffered saline to produce a scaffold ready for cell seeding.

Example 5-Evaluation of Proteoglycan Expression: The expression of proteoglycan (PG) molecules in each culture system is quantitatively assessed in terms of ^{35}S-sulfate incorporation rate into the chondrocytes culture according to the procedure by Morales *et al.*, *J. Bio. Chem.* 259 (Is. 11): 6720-29 (1984), incorporated herein by reference. To this end, ^{35}S-sulfate isotope (10μCi/ml) is added into each culture at forty-eight hours prior to the completion of culture period. At the completion of the culture, the conditioned media, cells, and polymer are collected and papain-digested at 60°C for 48 hours. A 200-microliter aliquot of each sample then is eluted on the PD-10 columns of Sephadex G-25M (Pharmacia,

Piscataway, NJ) and each fraction is collected in scintillation vials. The radioactivity of newly synthesized PG is counted with a scintillation counter and normalized with respect to the total DNA content per sample.

Example 6-Evaluation of Collagen Production: The collagen production of each culture is evaluated in terms of the specificity for collagen type I and II according to the method of Bonner *et al.*, *Eur. J. Biochem.* 46 (Is. 1): 83-88 (1974), incorporated herein by reference. To this end, ^{3}H-proline (15μCi/ml) with 50μg ascorbic acid is added into each culture at twenty-four hours prior to the completion of culture period. At the completion of culture, the conditioned media, cells, and polymer are collected and dialyzed against 0.05M NH_4HCO_3 at 4°C. The dry sample then is dissolved in SDS buffer and loaded onto a separating gel (6% polyacrylamide) topped with stacking gel (4.5% polyacrylamide) under a constant current supply of 30mA. The gel then is stained with Coomassie blue, destained overnight in a mixture of 10% methanol and 10% acetic acid, dried on a vacuum heater, then autoradiographs are taken. An appropriate collagen standard is used to identify the coexistence of types I and II collagen as detailed in the method of Scott *et al.*, *Conn. Tiss. Res.* 4: 107-16 (1976), incorporated herein by reference. In this method, CnBr-derived peptide from the specimens are analyzed on SDS-PAGE against the peptide standards obtained from the digested sample of purified types I and II collagen.

Example 7-Total DNA content: Two hundred microliters of papain-digested specimen per sample are aliquoted, and the fluorescent intensity of each sample is measured with a fluorescence spectrophotometer (Perkin-Elmer 650-10S) using the fluorometric reaction method according to Labarca *et al.*, *Anal. Biochem.* 102: 344-52 (1980), incorporated herein by reference, and Kim *et al.*, *loc. cit.* 174: 168-76 (1988), incorporated herein by reference, with Hoechst 33258 (Hoefer Scientific Inst., San Francisco, CA). The total DNA content of each sample is measured in units of μg/ml by using a calibration standard curve obtained with calf thymus DNA (D1501, Sigma, St. Louis, MO).

Example 8-Chondrocyte Culture on the Chitosan-GAG Membrane

The viability and metabolic activities of bovine metacarpophalangeal chondrocytes are cultured on thin membranes of CSA-augmented chitosan hydrogel. Chondrocytes cultured on

polystyrene 6-well culture plates are used as a control. The cell viability of the two culture systems is confirmed using a calcein-AM fluorescence labeling method as described in the manual of calcein-AM from Molecular Probes, Eugene, OR. Proteoglycan expression is determined at one week using ^{35}S-sulfate isotope incorporation. Chondrocyte expression of collagen type II expression is confirmed after 1 week with antibody staining. Chondrocytes cultured on polystyrene displayed a fibroblast morphology almost immediately, whereas chondrocytes cultured on CSA-chitosan hydrogel maintained chondrocyte-like round morphology for up to 7 days. After one week, chondrocytes cultured on the CSA-augmented chitosan membrane demonstrated a good cell viability as well as a significant increase in ^{35}S-sulfate incorporation rate as compared to the regular polystyrene plate culture, and continued to produce mostly collagen type II.

Example 9-*In Vivo* Chondrocyte Transplantation Using Chitosan-GAG Encapsulation

A total of forty-eight young adult male New Zealand White Rabbits, each weighing approximately three kilograms, are used for the *in vivo* cartilage repair experiment. Preoperatively, antibiotic prophylaxis of Mandol 500mg is administered subcutaneously. After induction of anesthesia with intravenous injection of Nembutal 30mg/kg, both rabbit knees are shaved, prepped, and draped in a sterile fashion. Bilateral arthrotomies are performed on each rabbit using the medial parapatellar approach. The patella is dislocated laterally, and the knee is slightly flexed to expose the articular cartilage of the medial femoral condyle. Using a drill punch and a scalpel, a partial-thickness chondral defect (3mm in diameter) is created in each medial femoral condylar surface without invading the subchondral bone. The defect is repaired as described below.

The four animal groups corresponding to the repair procedure performed are as follows: Group A) chondral defect filled with chitosan-GAG capsules with chondrocytes; Group B) chondral defect filled with chitosan-GAG beads without chondrocytes; Group C) empty chondral defect; Group D) sham surgery. In Groups A, B and C, the chondral defect area are sealed with a thin layer of fibrin-glue. The patella is then relocated and the incision suture closed in two layers using 5-0 vicryl. After surgery, recovery from anesthesia is monitored and rabbits are started on Torbutrol analgesic regimen (0.1 mg/kg IM BID x 3 days, then 0.1 mg/kg IM BID prn decreased cage activity or feeding). All rabbits are allowed to ambulate freely without any splintage.

Each group is comprised of n-12 rabbits (24 knees), and is divided into three subgroups of 4 rabbits (8 knees) corresponding to the time points of postoperative evaluation (6, 12, and 24 weeks, respectively). After the animals are sacrificed by intravenous injection of Pentothal, 8 knees in each subgroup are divided into two sets; two knees for histological evaluation, and six knees for biochemical evaluation.

Example 10-Histological Evaluation: Two knee specimens from each of the three subgroups corresponding to time points 6, 12, and 24 weeks postoperative are evaluated for histology (a total of 24 knees in the four surgical groups). At the time of sacrifice, cartilage specimens are immediately fixed in 10% formalin and decalcified in EDTA. Thin (1 micrometer) sagittal sections of cartilage tissue are stained with both safranin-O and hematoxyline and eosin. Two independent, blinded observers graded the tissue, according to methods described by O'Driscoll *et al.*, *J. Bone Jt. Surg.* 70-A: 595-606 (1988), and Amiel *et al.*, *loc. cit.* 67A: 911-20 (1985), the contents of each are incorporated herein by reference. The results are based on the following characteristics:

(a)Integrity of the interface of repaired & adjacent cartilage (fibrous vs. hyaline)

(b)Thickness

(c)Surface level of the repaired cartilage

(d)Surface regularity (*i.e.*, presence of fibrillation and fissuring)

(e)Staining quality and intensity of the matrix

(f)The cell morphology and population

(g)The orientation of collagen fibrils

Example 11-Biochemical Evaluation: Six knee specimens from each of the three subgroups corresponding to time points 6, 12, and 24-weeks postoperative are evaluated for biochemical composition (a total of 72 knees in the four surgical groups) immediately after sacrifice.

One third of the repaired cartilage specimens is used to determine the total water content of the cartilage. Immediately after the cartilage is excised from the bone plug, the wet-weight was measured. The tissue then is lyophilized for dry-weight measurement. Water content of cartilage is calculated as

Another one third of the tissue is used for collagen typing. Identification of the collagen type I and II peptides is obtained from CnBr digested samples of purified types I and II collagen. The CnBr peptides are identified by sodium dodecyl sulfate gel electrophoresis

on 10% polyacrylamide according to the method described by Scott *et al., supra.* A quantitative estimation of collagen typed content in the tissue then is determined by a video densitometer (Bio-rad, Model 620).

The remaining one third of the tissue is used for determination of total glycosaminoglycan content. Tissue is digested with papain solution (1mg/ml) at 65°C for 1 hour. The concentration of glycosaminoglycan is measured with a 1,9-dimethylmethylene blue binding assay according to the method of Farndale *et al., Biochem. Biophys. Acta* 883: 177-83 (1986), incorporated herein by reference.

Example 12-Determination of Physical Characteristics

A. Pore size

In determining the suitability of the inventive biomaterial for chondrocyte culture, several tests have been performed with the scaffold of the inventive biomaterial. Pore size was examined. Appropriate pore size facilitates mass transfer of the developing tissue, and serves as a void for appositional matrix growth of extracellular matrix. Pore size has been determined by histological analysis to range from 50 to 250μm.

B. Mechanical Properties: Biomechanical evaluation of the inventive scaffolds was determined.

A 2% chitosan scaffold solution, as prepared above, was extruded into a freezing mold, quick frozen in a 150°F freezer and then lyophilized for 24 hours.

An indentation test was performed on each scaffold using a 4mm-in-diameter indenter under 50% strain and the reaction force was measured on each specimen. The data showed that chitosan reinforced with PGA/PLLA produced a higher reaction force than the chitosan-alone scaffold, thereby indicating that the chitosan-PGA/PLLA composite scaffold exhibits additional structural stiffness.

Example 13-In vitro culturing: Chondrocytes were cultured in-vitro on the inventive PGA/PLLA-chitosan composite matrix. Fresh articular cartilage was obtained from the metacarpophalangeal joints of young (1-2 year old) bovine specimens. Within four hours of sacrifice, chondrocytes were isolated from the tissue and seeded onto the CSA-augmented (DMEM w/10% FBS, 1% penicillin/streptomycin). After one week culture, the matrices were fixed in glutaraldehyde and subjected to critical point drying protocol in preparation for

Scanning Electron Microscop (SEM). The SEM of our scaffold showed excellent phenotype retention by the seeded chondrocytes. The chondrocytes did not dedifferentiate into fibroblasts, but stayed round. Additionally, the scaffold was fairly homogenous and presented consistent morphology across specimens.

Example 14-Different Structural Forms for in vitro Culturing: Three different structural forms of GAG-augmented chitosan-based hydrogel were tested for chondrogenesis in terms of cell viability, synthesis of matrix molecules (collagen and proteglycan), and cartilage-specific genes (aggrecan, biglycan, decorin, and collagen types II, IX, XI) expression. First, bovine chondrocytes were harvested from the metacarpophalangeal joint of a young bovine specimen. Chondrocytes from primary culture or passage 3 culture were then seeded on the surface of a thin membrane (~0.1mm thick) made of CSA-augmented chitosan, seeded in a 3-dimensional porous scaffold of polyglycolic/polylactic acid (PGA/PLLA)-CSA chitosan composite, or microencapsulated in CSA-chitosan beads. Five μCi/ml of ^{35}S-sulfate or ^{3}H-proline were added to the culture systems for 24 hours at one or two weeks of culture, and analyzed for the proteglycan and collagen. Chondrocytes cultured on the above three structural chitosan-based materials demonstrated superb chondrogenesis-supporting characteristics with a 4 to 8-fold increase in proteoglycan synthesis, as compared to a conventional monolayer culture on a polystyrene dish.

Example 15-Addition of TGF-β1: TGF-β1 (0.5 ng/ml or 2.0ng/ml) was also added to the culture systems to investigate it dose-dependent simulative effect on the synthesis rate of proteglycans and collagen.

By adding TGF-β1 to the culture system, matrix synthesis was enhanced. Collagen synthesis was also increased by growing chondrocytes on CSA-augmented chitosan with an average increase of 66% over control cells with additional increase in collagen production averaging 177% for 0.5 ng/ml TGF-β1. Additionally, the results suggest that CSA-augmented chitosan may potentiate the re-differentiation of passaged chondrocytes.

WHAT IS CLAIMED IS:

1. A construct comprising chondrocytes and a chitosan-based composite material that is complexed with a glycosaminoglycan molecule.

2. The construct of claim 1, wherein said glycosaminoglycan is selected from the group consisting of, heparan sulfate, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, dermatan sulfate, keratin sulfate, hyaluronic acid, and heparin.

3. The construct of claim 1, which is in the form of a hydrogel.

4. The construct of claim 1, which is a film.

5. The construct of claim 1, which is a three-dimensional scaffold.

6. The construct of claim 1, which is a microcapsule.

7. The construct of claim 1, which is in the shape of a chondral defect site.

8. The construct of claim 1, wherein the chitosan-based composite material comprises a composite of chitosan and polyglycolic acid/poly-l-lactic acid.

9. A method of transplanting chondrocytes to a patient, comprising placing chondrocytes in a construct comprising a chitosan-based composite material that is complexed with a glycosaminoglycan molecule and placing said construct containing said chondrocytes in said patient.

10. The method of claim 9, wherein said glycosaminoglycan is selected from the group consisting of heparan sulfate, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, dermatan sulfate, keratin sulfate, hyaluronic acid, and heparin.

11. The method of claim 9, wherein said construct is in the form of a hydrogel.

12. The method of claim 9, wherein said construct is a film.

13. The method of claim 9, wherein said construct is a three-dimensional scaffold.

14. The method of claim 9, wherein said construct is a microcapsule.

15. The method of claim 9, wherein said construct is in the shape of a chondral defect site.

16. The method of claim 9, wherein said chitosan material comprises a composite of chitosan and polyglycolic acid/poly-l-lactic acid.

17. A method of repairing damaged cartilage, comprising administering to the site of said damage, a therapeutically effective amount of chondrocytes in a construct of a chitosan-based composite material that is complexed with a glycosaminoglycan molecule.

18. The method of claim 17, wherein said construct comprises glycosaminoglycan is selected from the group consisting of, heparan sulfate, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, dermatan sulfate, keratin sulfate, hyaluronic acid, and heparin.

19. The method of claim 17, wherein said construct is a hydrogel.

20. The method of claim 17, wherein said construct is a film.

21. The method of claim 17, wherein said construct is a three-dimensional scaffold.

22. The method of claim 17, wherein said construct is a microcapsule.

23. The method of claim 17, wherein the chondrocytes are administered via open arthrotomy.

24. The method of claim 17, wherein the damaged cartilage is articular cartilage.

25. The method of claim 17, wherein said chitosan material comprises a composite of chitosan and polyglycolic acid/poly-l-lactic acid.

26. A method of promoting chondrocyte-specific phenotypes using a carrier material comprising a chitosan-based composite material that is complexed with a glycosaminoglycan molecule.

27. The method of claim 26, wherein said carrier material is a film.

28. The method of claim 26, wherein said carrier material is a microcapsule.

29. The method of claim 26, wherein said carrier material is a three-dimensional scaffold.

30. The method of claim 26, wherein the carrier material is a hydrogel.

31. A method of preparing the biodegradable construct of claim 1, comprising (a) bringing an acetic acid solution of a chitosan-based composite material into contact with a glycosaminoglycan-containing solution, such that the construct is formed, and then (b) adding chondrocytes to the construct.

32. The method of claim 31, wherein the chitosan-based composite material comprises a composite of chitosan and polyglycolic acid/poly-l-lactic acid.

33. The method of claim 31, wherein the chitosan dissolved in acetic acid is placed in a pre-molded glass container and frozen.

34. The method of claim 33, wherein the pre-molded glass container is in the shape of the chondral defect site.

35. The method of claim 31, further comprising lyophilizing.

36. The method of claim 31, wherein the chondrocytes are harvested and cultured.

INTERNATIONAL SEARCH REPORT

Inte. ional Application No
PCT/US 99/05887

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L27/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 8735 Derwent Publications Ltd., London, GB; Class A96, AN 87-245769 XP002108746 & JP 62 166891 A (SNOW BRAND MILK PROD CO LTD), 23 July 1987 (1987-07-23) abstract ---	1-3,6
P,X	DENUZIERE A ET AL: "Chitosan-chondroitin sulfate and chitosan-hyaluronate polyelectrolyte complexes: biological properties" BIOMATERIALS, vol. 19, no. 14, July 1998 (1998-07), page 1275-1285 XP004161388 ISSN: 0142-9612 the whole document --- -/--	1-7, 9-15, 17-24, 26-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 1999	20/07/1999

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter .onal Application No
PCT/US 99/05887

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 96 02286 A (CYTOTHERAPEUTICS INC) 1 February 1996 (1996-02-01) page 9, line 3; claims	1-36
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A	WO 90 12603 A (VACANTI CHARLES A ;LANGER ROBERT S (US); VACANTI JOSEPH P (US)) 1 November 1990 (1990-11-01) claims; examples 1-5	1-36
A	EP 0 296 078 A (CENTRE NAT RECH SCIENT) 21 December 1988 (1988-12-21) claims; example 5	1-36

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 05887

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 9-25 are directed to a method of treatment of the human/ animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. .ional Application No
PCT/US 99/05887

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